

Shoot organogenesis and plant regeneration in *Metabriggsia ovalifolia*

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Abstract An efficient propagation and regeneration system via direct shoot organogenesis for an endangered species, *Metabriggsia ovalifolia*, was established. High activity cytokinins [6-benzyladenine (BA) and thidiazuron (TDZ)] and low activity auxins [α -naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA)] could directly induce adventitious shoots from leaf or petiole explants within 5 weeks. Cytokinins (TDZ or BA) combined with auxin (NAA) in the induction media induced more adventitious shoots than when auxins or cytokinins were used alone. Adventitious shoots could be induced and also mass-propagated on media containing 2.5–5.0 μ M TDZ (or BA) and 0.25–0.5 μ M NAA. Adventitious roots differentiated at the proximal end of shoots on rooting media containing half-strength MS salts and 0.5 μ M IBA, 0.5 μ M NAA, 0.1% activated charcoal or no plant growth regulators. Over 90% of plantlets survived following acclimatization and transfer to a potting mixture (1:1, sand:vermiculite) in basins.

Keywords *Metabriggsia ovalifolia* · Shoot organogenesis · Mass propagation · Plant regeneration

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Abbreviations

2, 4-D	2, 4-Dichloro-phenoxyacetic acid
BA	6-Benzyladenine
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KIN	Kinetin
NAA	α -Naphthaleneacetic acid
TDZ	Thidiazuron

Introduction

The family Gesneriaceae contains 150 genera and 3,700 species in the world. They are distributed mainly in tropical to temperate zones of eastern and southern Asia, Africa, Oceania and South America to Mexico (Weber 2004). China has 58 genera and 463 species, among which 27 genera and 375 species are endemic; more than half the species exist in narrow areas (Li and Wang 2004). Most species have ornamental value, such as African violets, and many species contain important compounds (such as flavonoids and glucosides, among others) (Harborne 1967; Damtoft and Jensen 1994; Terreaux et al. 1996; Griesbach 1998; Liu et al. 1998); so they have great medicinal value. Many species have great conservation and scientific value (Podolsky 1992; Ma et al. 2010a). *Metabriggsia ovalifolia* W.T. Wang is a perennial herb and it is endemic solely to Guangxi Province in China (Wang 1983). In China, only four species in the Gesneriaceae family have been listed as endangered, *M. ovalifolia* being one of them, and this is due to reduced size and distribution of this population (Lu et al. 1989; Wang and Xie 2004). Chromosome number ($2n = 24$) and geographic distribution have been

assessed (Li 1996; Cao et al. 2003). However, studies on basic biology and primary endangered mechanisms have not yet been reported. In order to preserve and utilize rare and endangered plant species (e.g., *Ochna integerrima*; Ma et al. 2010b), it is essential to establish an efficient propagation and plant regeneration system in the event of sudden deterioration or loss of the natural environment. Since there have been no reports so far on tissue culture or biotechnology of *M. ovalifolia*, our current study thus focuses on the effects of different plant growth regulators (PGRs) on the induction of organogenesis for the establishment of an efficient propagation and plant regeneration method.

Materials and methods

Establishment of in vivo mother plants

Plants of *M. ovalifolia*, originally growing at 600–1,200 m in mountain areas of Hechi, Guangxi Province, were brought back alive to the South China Botanical Garden, Guangzhou, and then transplanted into several basins in March 2009. The basins were 20 cm high and 30 cm in diameter and contained a mix of sand, ocher and humus soil (1:1:1). The plants were kept alive under a half-shade shed for growth under natural conditions.

Influence of PGRs on shoot organogenesis from leaf explants

Young leaves from in planta mother plants were used as initial explants, washed in running tap water for 10 min. Leaf surfaces were cleaned with absorbent cotton soaked in 70% alcohol for 30 s and immersed and stirred in 0.1% mercuric chloride for 10 min, and then rinsed 3 times with sterile distilled water. After that, they were placed on paper towels and allowed to dry naturally. Then, they were cut into explants 0.4 cm² in size and inoculated onto MS basal media (Murashige and Skoog 1962) supplemented with the same concentration (2.5 μM) of PGRs [6-benzyladenine

(BA), thidiazuron (TDZ); kinetin (KIN), 2,4-dichlorophenoxyacetic acid (2,4-D), α -naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA)] for morphogenic induction (Table 1). Every treatment contained 30 explants which were divided between 5 jars. The culture jars were 10 cm high and 6 cm in diameter. Experiments were repeated twice. All media contained 30 g l⁻¹ sucrose and were adjusted to pH 5.8 and solidified with 0.6% agar and placed for 2 weeks in the dark. After dark incubation, cultures were transferred to light with a 10-h photoperiod at a photon flux of 80 μmol m⁻² s⁻¹ in a culture room at 25 ± 1°C. After a total of 5 weeks culture, induction of shoot organogenesis was investigated and compared. All data were statistically analyzed using the LSD test ($P = 0.05$).

Effect of PGR combinations on induction of shoot organogenesis from leaf and petiole explants in vitro

Clumps with adventitious shoots from the above treatments were transferred to MS medium containing no PGRs for shoot development. Leaves and petioles from in vitro plantlets derived from the single PGR experiments were then used as explants; they were cut into explants 0.5 cm² in area or 1.0 cm in length, respectively, and inoculated onto media containing different concentrations and combinations of PGRs (Table 2), then transferred to the dark for 2 weeks. Dark-adapted explants were transferred to light with a 10-h photoperiod at a photon flux of 80 μmol m⁻² s⁻¹, as for the single PGR experiments. Every treatment contained 60 explants that were inoculated in 10 jars. Experiments were repeated twice. After 5 weeks in culture, induction of organogenesis was investigated and compared. All data were statistically analyzed using the LSD test ($P = 0.05$).

Shoot organogenesis and shoot proliferation

Two methods to propagate shoots were assessed. The first was conventional shoot propagation: clumps with adventitious shoots (20–40 shoots) were divided into smaller

Table 1 Effect of plant growth regulators on shoot organogenesis from leaf explants of *Metabriggsia ovalifolia*

PGR (μM)	Observation results on the leaf explants	Mean number of adventitious shoots per leaf explant
0	Maintained green, a few shoots	2.3 d
2.5 BA	No callus, adventitious shoots	25.6 b
2.5 TDZ	Callus, adventitious shoots	36.7 a
2.5 KIN	No callus, no shoots, yellow and necrosis	0 e
2.5 IBA	Callus, adventitious roots, adventitious shoots	7.5 c
2.5 NAA	Adventitious roots, callus, adventitious shoots	5.8 c
2.5 IAA	Callus, adventitious shoots, adventitious roots	8.7 c
2.5 2,4-D	Callus, no shoots, necrosis	0 e

Means with the same letter within a column are not significantly different. LSD tests at $P = 0.05$

Table 2 Effects of PGRs and explants in vitro on shoot organogenesis in *M. ovalifolia*

PGR combinations (μM)	Mean number of adventitious shoots per explant	
	Leaf	Petiole
1.0 BA + 0.1 NAA	42.1 c	46.0 b
2.5 BA + 0.25 NAA	45.0 c	53.0 b
5.0 BA + 0.5 NAA	60.5 b	69.4 a
1.0 TDZ + 0.1 NAA	61.0 b	36.0 b
2.5 TDZ + 0.25 NAA	61.5 b	41.5 b
5.0 TDZ + 0.5 NAA	79.1 a	67.1 a
1.0 KIN + 0.1 NAA	17.0 d	10.9 c
2.5 KIN + 0.25 NAA	21.3 d	13.4 c
5.0 KIN + 0.5 NAA	30.0 d	16.7 c

Means with the same letter within a column are not significantly different. LSD tests at $P = 0.05$

Table 3 Comparison of axillary shoot proliferation versus shoot organogenesis from leaf tissues after 5 weeks

PGR combinations (μM)	Mean number of shoots per explant	
	Axillary shoot proliferation	Leaf shoot organogenesis
2.5 BA + 0.25 NAA	22.5 a	53.0 c
5.0 BA + 0.5 NAA	23.6 a	69.4 b
2.5 TDZ + 0.25 NAA	27.8 a	61.5 b
2.5 TDZ + 0.25 NAA	29.4 a	79.1 a

Means with the same letter within a column are not significantly different. LSD tests at $P = 0.05$. All treatments included 1/2 MS medium

clumps (3–4 shoots) and then inoculated onto different media for further propagation (Table 3). Every treatment included 5 clumps per culture jar, with a total of 40 clumps. The culture jars were 10 cm high and 6 cm in diameter. The efficiency of propagation was assessed by mean increment times after culture in the light for 5 weeks. Simultaneously, a second method was employed to induce adventitious shoots from immature leaf explants in vitro. In this case, petioles or leaves were cut into explants 1 cm in length or 0.5 cm² in area, respectively, and then inoculated into culture jars on different induction media (Table 3). After culture for a total of 5 weeks, the efficiency of propagation was assessed by mean increment times.

Root formation and acclimatization

When the shoots reached 1–3 cm in height, they were transferred to half-strength MS medium supplemented with 0.5 μM NAA, 0.5 μM IBA, 0.1% activated charcoal or no

PGRs, respectively, for root formation. The shoots were inoculated into culture jars 10 cm high and 6 cm in diameter. Then, 2 and 4 weeks later, root formation was investigated. After culture for a total of 45 days in the media, plantlets with 4–6 leaves and 2–4 cm high were removed from the jars and the agar was gently removed by rinsing in water, then 100 plantlets from each treatment were transplanted to a potting mixture (1:1, sand:vermiculite) under half-shade in a shed where they were sprayed with water once a day and watered with half-strength MS solution (only macro- and microelements) once a week. Plant survival was then quantified after 4 weeks.

Results

Effect of single PGR on shoot organogenesis from leaf explants

The leaf explants showed no response on PGR-free medium within 3–4 weeks. After that, some small protuberances with very little callus were visible on the leaf surface or cut surfaces. Thereafter, some adventitious shoots developed from the callus (Table 1).

The leaf explants showed no response on medium containing 2.5 μM BA in the first 2 weeks of the culture period. However, 2 weeks later, i.e., by the 4th week, leaf explants began to become obviously swollen and generally some protuberances occurred directly at the leaf surface or leaf blade. After culture for a total of 5 weeks, some adventitious shoots were obviously visible (Fig. 1a).

On medium containing 2.5 μM TDZ, leaf explants seemed to show no differences with respect to the previous treatments. However, 2 weeks later, the leaf explants generally became much more swollen, and then some callus was visible on cut surfaces and on the leaf lamina. After culture for a total of 5 weeks, some adventitious shoots were obviously visible (Fig. 1b). TDZ-containing medium induced more adventitious shoots than medium containing 2.5 μM BA (Table 1).

For the medium containing 2.5 μM KIN, the leaf explants became yellow, then generally necrotic, and no adventitious shoots were visible.

When the leaf explants were cultured on medium containing 2.5 μM 2,4-D, some callus was induced on the cut surface within 2 weeks. However, the callus could not develop shoots within 5 weeks. The callus generally became brown and necrotic.

On medium containing 2.5 μM IAA, leaf explants always remained green and there was no response within 3 weeks, after which some protuberances with a few adventitious shoots developed directly on the leaf surface

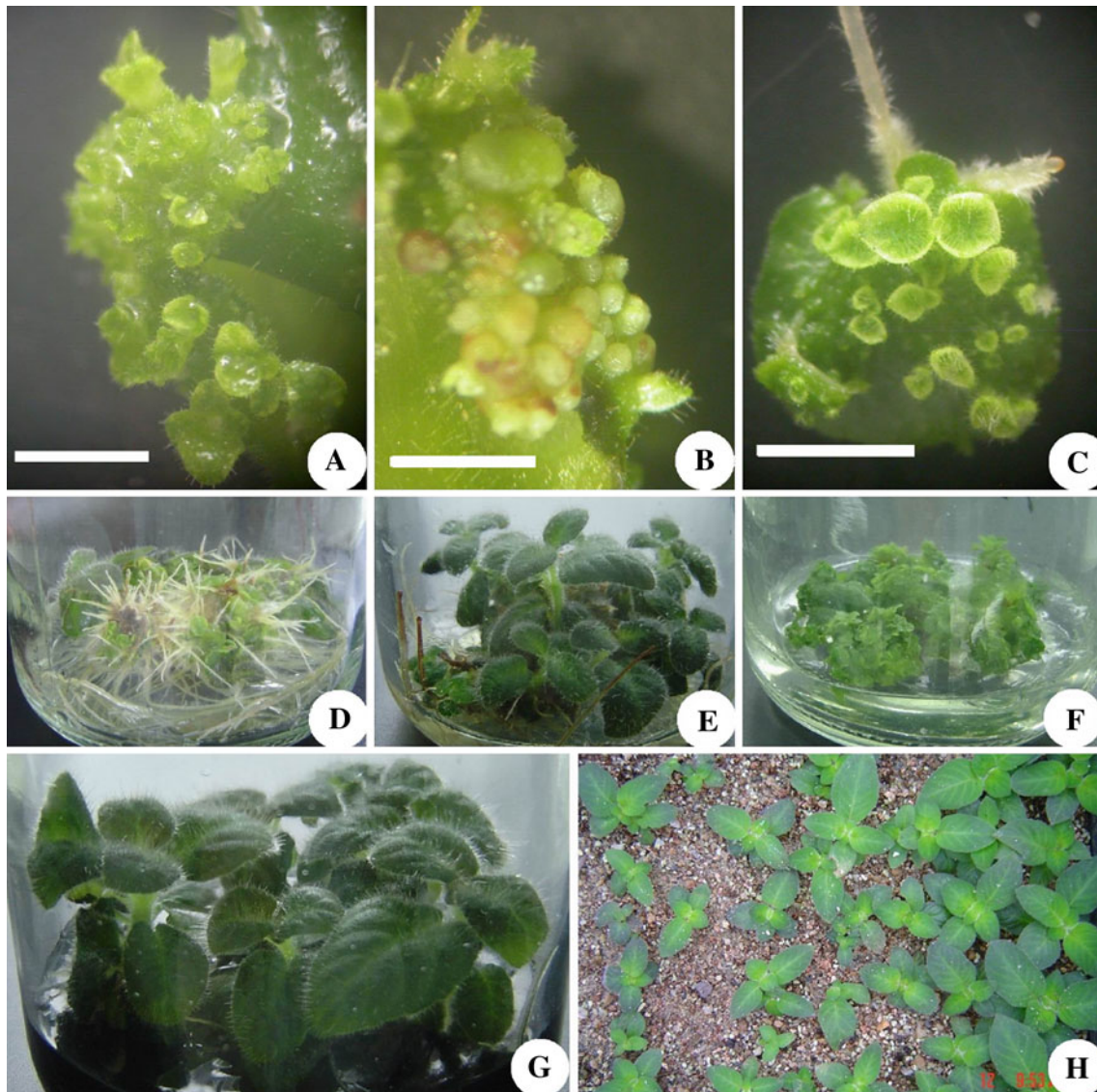


Fig. 1 Shoot organogenesis and plant regeneration in *Metabriggsia ovalifolia* (bars 2 mm). **a** Adventitious shoots developed from immature leaf explants on induction medium containing 2.5 μ M BA after culture for 35 days; **b** adventitious shoots developed from immature leaf explants on induction medium containing 2.5 μ M TDZ after culture for 35 days; **c** adventitious roots and then adventitious shoots developed from immature leaf explants on induction medium containing 5.0 μ M IAA after culture for 32 days; **d** adventitious roots and then adventitious shoots developed from immature leaf explants

on induction medium containing 2.5 μ M NAA after culture for 35 days; **e** shoots grew up and adventitious roots formed on PGR-free medium after culture for 45 days; **f** immature leaf explants were cultured on induction medium containing 2.5 μ M TDZ after culture for 45 days; **g** roots were induced on MS medium containing 0.1% activated charcoal free of PGRs. **h** more than 90% of plantlets survived after being transplanted for 30 days in a mixture of sand and vermiculite in basins

or on cut surfaces (Fig. 1c). Then, some adventitious roots also developed within 2 weeks.

Other auxins (2.5 μ M NAA and 2.5 μ M IBA) could induce little callus. In those cases, some adventitious roots were first visible on the callus or directly on the leaf surface. After a total of 4–5 weeks in culture, some adventitious shoots developed among the rooted leaf explants (Fig. 1d). The number of shoots induced was much less than when TDZ or BA was used (Table 1).

Effect of PGR combinations on induction of shoot organogenesis from leaf and petiole explants in vitro

When clumps with adventitious shoots were transferred to PGR-free MS medium for further culture, shoots developed quickly. They would grow 2–3 cm high and their leaves could develop 1–2 cm in length within 1 month. In the same time frame, some adventitious roots also formed at the base of shoots (Fig. 1e).

When the leaf and petiole explants *in vitro* were cultured for a total of 4–5 weeks in induction media, adventitious shoots became visible (Table 2). Both explants could induce adventitious shoots. Among them, the highest number (79.1) of mean shoots induced from leaf explants was that containing 5.0 μM TDZ and 0.5 μM NAA and the highest number (69.4) of mean shoots induced from petiole explants was that containing 5.0 μM BA and 0.5 μM NAA. However, any combination with KIN and NAA induced fewer shoots than the above media, indicating that TDZ and BA have a stronger induction effect than KIN. Somatic embryogenesis was never observed from either explant.

Shoot organogenesis and shoot proliferation

After 5 weeks culture, the number of axillary shoots reached 22–29 in any of the four media while the number of leaves formed was 53–79 in the same media (Table 3). Thus, the first method, i.e., induction of shoot organogenesis from leaf explants, is much quicker than the normal shoot propagation method. Despite this, the shoots in the normal shoot propagation method could develop quickly whereas shoots in the first method never developed (Fig. 1f). This factor needs to be considered when assessing plant regeneration and acclimatization.

Root formation and acclimatization

When shoots were transferred to regeneration media for root formation (Table 4), media containing 0.5 μM IBA, 0.5 μM IAA or 0.5 μM NAA could develop roots within 2 weeks indicating that auxins enhanced root formation (Fig. 1g). The other two treatments could also develop roots within 4 weeks. As the plantlets were transferred to basins containing a mixture of sand and vermiculite, usually >90% of the plantlets could survive. Among them, plantlets 4 cm high recorded the highest survival (97.5%), while

plantlets 2 cm high exhibited a lower survival rate (91.5%); no obvious aberrant plants were found (Fig. 1h).

Discussion

In the family Gesneriaceae, most tissue culture usually originates from leaf explants. Somatic embryogenesis or shoot organogenesis has been successfully induced in some species: in *Chirita medica*, adventitious buds were induced from leaf explants on medium with 0.1 mg l^{-1} NAA and 0.1 mg l^{-1} BA (Li et al. 2009); in *Aeschynanthus radicans*, 40% of leaf explants produced somatic embryos when induction medium contained 6.81 μM TDZ and 2.68 μM 2,4-D (Cui et al. 2009). In *Sinningia speciosa*, leaf explants were cultured on MS medium containing 2.0 mg l^{-1} BA and 0.2 mg l^{-1} NAA, and most explants produced small green calli, and then adventitious buds formed (Xu et al. 2009). For *Primulina tabacum* Hance, TDZ induced somatic embryogenesis and BAP induced shoot organogenesis, and TDZ and BA in combination could induce both somatic embryogenesis and shoot organogenesis from leaf explants (Ma et al. 2010a). There are many reports of shoot organogenesis and somatic embryogenesis and gene transformation via leaf explants of the ornamental plant, African violet (Mølgaard et al. 1991; Ohki 1994; Lo 1997; Lo et al. 1997; Mercuri et al. 2000). All these studies indicated that different species need different PGRs combinations and concentrations and, in all cases, the regeneration pathway maybe either somatic embryogenesis, shoot organogenesis, or both. TDZ is able to alter the pathway of morphogenesis from shoot organogenesis to somatic embryogenesis through a simple change in TDZ concentration (Mithila et al. 2003; Ma et al. 2010b). Buds on *Kalanchoe pinnata* leaf discs treated with 10^{-6} M TDZ produced compact, dwarf and rootless plantlets having achlorophyllous leaves with increased number of marginal notches (Jaiswal and Sawhney 2006). In *Paphiopedillum* orchid hybrid PH59, 4.54 μM TDZ increased the number of shoots per explant when leaf segment explants were used, while in another hybrid, PH60, 4.52 μM 2,4-D + 0.45 μM TDZ promoted direct shoot bud formation from leaf segment explants (Chen et al. 2004). A commonly observed phenomenon, namely the induction of callus from TDZ treatments, was observed in young leaf explants of dayleaf lily (*Hemerocallis*) which could be induced to form callus in the presence of 4.55 or 6.81 μM TDZ (Li et al. 2010).

In our study, however, somatic embryogenesis was not induced from leaf explants of *M. ovalifolia*. Shoot organogenesis was only induced from leaf and petiole explants on different media with various PGRs, singly or in combination. Even no PGRs or weak concentrations of auxins

Table 4 Influence of different auxins and activated charcoal on rooting and plantlet survival of *M. ovalifolia*

Treatment (μM)	Culture time		Root formation in 4 weeks (%)	Survival of transplanted plants (%)
	2 weeks	4 weeks		
0.5 IBA	+	+	100 a	95.5 a
0.5 NAA	+	+	100 a	93.8 a
0.5 IAA	+	+	100 a	95.2 a
0	–	+	100 a	89.5 a
0.1% activated charcoal	–	+	100 a	90.4 a

+ indicates root formation, and – indicates roots were not formed yet within the observed time period. Means with the same letter within a column are not significantly different. LSD tests at $P = 0.05$

(IAA, IBA and NAA) or cytokinins (BA and TDZ) could induce some adventitious shoots. This indicates that shoot organogenesis from leaf or petiole explants in *M. ovalifolia* is not that dependent on PGR type. The auxins IAA, IBA and NAA were used for enhancing root formation. Oddly, these auxins could not only enhance root formation of plantlets but also induce both adventitious root and shoot formation from leaf explants (Table 1 and 4; Fig. 1c, d). They are seldom successfully used for induction of somatic embryogenesis or shoot organogenesis and there is only one report on cassava somatic embryogenesis from immature leaf explants being induced by a high concentration of NAA (40 mg l⁻¹) or 2,4-D (4.0 mg l⁻¹) (Ma and Xu 2002). However, in *M. ovalifolia*, the low activity auxins succeed in the induction of shoot organogenesis at very high frequencies whereas a strong auxin (such as 2,4-D) could not successfully induce shoot organogenesis. The more highly active cytokinins such as BA and TDZ could induce a high efficiency of adventitious shoots from leaf explants of *M. ovalifolia* although the low activity cytokinin KIN could not. This was an opposite trend to the auxins: lower activity auxins (IAA, IBA and NAA) could induce adventitious shoots from leaf explants of *M. ovalifolia* but the high activity auxin 2,4-D could not. So, in the tissue culture of *M. ovalifolia*, high activity cytokinins and low activity auxins could enhance induction of adventitious shoot formation. The combination of cytokinins and auxins in induction media enhanced induction frequency. The possibility for using a wide range of PGR types and combinations will ensure the establishment of efficient induction and regeneration systems in *M. ovalifolia*.

Some regenerated and acclimatized plants have been transplanted to high-lying mountains and the polar region conservatory in South China Botanical Garden and in its original habitat. Most of the plants could grow and develop well in all the above locations.

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